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Protocol

Synthesis of high avidity antibody fragments (scFv multimers) for cancer imaging

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Abstract

Multivalent antibody fragments (scFv dimers, trimers and tetramers) provide high avidity and ideal pharmacokinetics for tumour targeting applications. This protocol describes our optimised protocol for high-level bacterial synthesis of soluble antibody scFv fragments, as either monomers or multimers, using the heat-inducible bacterial expression vector pPOW3. Our protocol is rapid, which minimizes protein degradation, and utilises inexpensive reagents for cost-effective product synthesis. The strong, temperature-regulated promoters in pPOW3 provide efficient production of either monomeric or multimeric single-chain antibody fragments as dictated by the gene construct design.

Keywords: *E. coli*; Heat-induction; Soluble; Antibody; Multimers; scFv

1. Type of research

(1) This protocol describes our preferred method for high level synthesis of antibody fragments (scFv monomers or multimers) in the bacterial periplasm, utilising procedures for recovery by either osmotic shock or by denaturation and refolding. Purification is achieved by affinity chromatography using a tag tail such as the octapeptide (FLAG) fused to the C-terminus of the antibody construct, as described by Power et al. (1992).

(2) Single chain Fv (scFv) fragments provide the original affinity of the parent antibody and are synthesized efficiently in bacteria since the V_H and V_L domains are tethered together with a polypeptide linker (reviewed by Hudson and Kortt, 1999). Typically, scFv monomers (30 kDa) are designed with the C-terminal end of the V_H domain (defined as Ser^{112} using the numbering system of Kabat et al., 1991) tethered by a polypeptide linker to the N-terminal residue of V_L or, in inverse orientation, the C-terminal end of the V_L domain (defined as Arg^{107}) is tethered to the N-terminal residue of V_H (Malby et al., 1993, 1998). Owens and Young (1994) showed that these small scFv monomers have suitable tumour imaging properties due to rapid tissue pene-

Abbreviations: Ab, antibody; CDR, complementarity determining region; Fab, antibody fragment produced by proteolysis; Fv, complex of V_H and V_L domains; Ig, immunoglobulin; kDa, kilodalton; Mab, monoclonal antibody; M_r, molecular mass; PCR, polymerase chain reaction; scFv, single chain Fv molecule; V_H, variable region from antibody heavy chain; V_L, variable region from antibody light chain

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tration and blood clearance (reviewed by Colcher et al., 1998).

(3) When the polypeptide linker length is reduced to 3–12 residues it cannot fold into a functional Fv domain and instead associates with a second scFv molecule to form a diabody of ~60 kDa (Holliger et al., 1993; Kortt et al., 1994). Reducing the linker length to <3 residues forces scFv association into trimers (triabodies, ~90 kDa) or tetramers (~120 kDa) depending on linker length, composition and Fv domain orientation (Atwell et al., 1999; Le Gall et al., 1999). The increased binding valency in these scFv multimers results in high avidity (long off-rates). A significant advantage for tumor targeting is that molecules of ~60–100 kDa have increased tumor penetration and fast clearance rates compared to the parent Ig (150 kDa) (Adams et al., 1998; Hudson, 1999; Wu et al., 1999). A number of cancer-targeting scFv multimers have recently undergone pre-clinical evaluation for in vivo stability and efficacy (Adams et al., 1998; Wu et al., 1999).

(4) Our protocol describes vectors which utilise strong tandem lambda promoters with tight repression to ensure efficient cell/vector growth, followed by short burst of induction (Power et al., 1992; Kortt et al., 1995). Alternative vectors have been described (Skerra, 1994; Coia et al., 1996; Zhu et al., 1996) which also produce recombinant proteins in yields up to 1 g/l which represent over 50% of the total recoverable bacterial proteins. These expression systems supersede vectors with 'leaky' promoters (*lac*, *trp* and *tac*), all of which are not effectively repressed. Antibody fragments fold efficiently only in the oxidising extracellular environment, so are optimally targeted to the periplasm using secretion signals (most commonly *pel*B and *omp*A).

2. Time required

The time required to run the protocol (whole protocol) is 5 days. The time required for each step is as follows

1. Protein expression
   1.1. small scale screen to determine optimum induction time (2 days)
   1.2. shake flask expression (1 litre) and cell fractionation
      1.2.1. Preparation of starter culture (day 1)
      1.2.2. Induction of protein synthesis (day 2)
   1.3. Protein fractionation (days 3 and 4)
   1.4. Protein purification by affinity chromatography (day 5)

3. Materials

Special equipment

3.1. For protein expression

- Autoclave
- Shaking incubators at 30°C and 42°C
- 2-l baffled Erlenmeyer flasks
- Micropipettes covering the range 1μl to 1 ml
- Floor model (Beckman) centrifuge and rotors and bottles to spin at 3000–14 000 *g*
- 12% glycine PAGE gels (Gradipore)
- SDS protein sample dye
- Electrophoresis system (e.g. Bio-Rad mini protean system)
- Coomassie blue R-250
- destain: 50% methanol, 10% acetic acid
- 10% acetic acid
- Western blotting system and reagents (Bio-Rad)
- bench top orbital shaker (Raytek)

3.2. For protein fractionation

Ultra turrex homogeniser T25 (Janke & Kunkel)

3.3. For protein purification

- 5-litre non-baffled flask
- dialysis tubing 6–8 K MW cut-off (Spectrum Lab.)
- sintered glass funnel
- vacuum flask
- chromatography columns (e.g. Econo-columns, Bio-Rad)
- Spectrophotometer

3.4. For protein product analysis

- HPLC system
- Superose 12 column
- Chemicals and reagents

All chemicals are BDH Analar grade unless otherwise specified.

3.5. Bacterial expression vector

The pPOW3 temperature induced expression vector (available from the authors on request) comprises the features shown in Fig. 1. The vector encodes the F1 origin for designed point mutations (via phage production and site-directed mutagenesis using single-strand DNA). The lambda left and right promoters in tandem control expression of the inserted scFv gene. The promoters are controlled by the temperature sensitive repressor gene *cI*857. Protein synthesis is induced by heat-shock, which inactivates the repressor. Constitutive expression of the repressor gene from the plasmid allows a wide range of host cells to be used for expression. The 5′ cloning sites for antibody scFv genes are *Sfi*I, *Nco*I and *Msc*I in the *pelB* gene that encodes the signal sequence, (the N-terminal PelB signal sequence is removed by signal sequence peptidases immediately following secretion of the PelB-scFv fusion protein). The 3′ cloning sites for antibody scFv genes are *Bam*HI, *Eco*RI or *Sal*I sites, retaining the termination codons (one in each reading frame) in the *Eco*RI or *Sal*I sites (Fig. 2). There is no in-frame peptide tag-tail in the vector to aid in the protein purification so if desired this should be encoded within the reverse PCR primer before the termination codon. The plasmid contains the gene for ampicillin resistance.

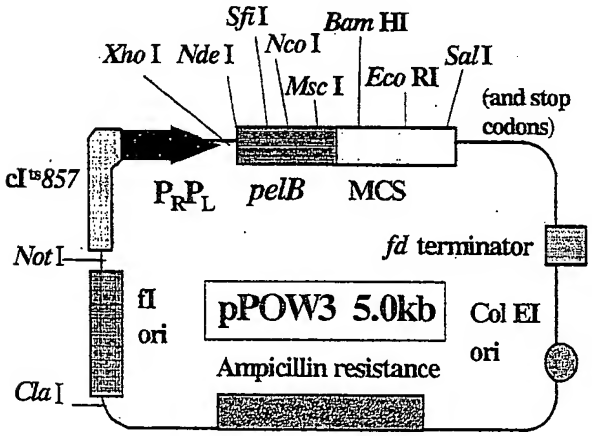


Fig. 1. pPOW3 Vector diagram and PelB secretion signal sequence, including unique cloning sites. This vector has an improved MCS (multiple cloning site) region to the original pPOW (16) containing *Sfi*I and *Nco*I cloning sites, translational stop codons, and an FI origin of replication. The vector is available from the authors on request.

3.6. Primers

General pPOW3 oligos for sequencing or insert size analysis by PCR.

N2175 pPOW3 forward sequencing primer. This primer binds 50 bp upstream of the *pelB* sequence, before the *Xho*I site. 5′ TGT GTG ATA CGA AAC GA 3′ (17 mer).

N2357 pPOW3 reverse sequencing primer. This primer binds 8 bp downstream of the multiple cloning sites. It is a reverse primer and it can be used to sequence any DNA that has been cloned into the multiple cloning sites. 5′ GCG CGT CGG GCT CTA GA 3′ (17 mer).

3.7. Bacterial cell growth media

Purified deionised water should be used for all solutions.

2×YT+Amp: 8.0 g tryptone, 5.0 g yeast extract, 5.0 g NaCl, (for agar plates add 15 g Bacto agar); make to 1 litre with water. Autoclave and cool then add Ampicillin to 100 μg/ml. Alternative bacterial cell growth SB (superbroth) media can be used (see Section 6.3).

Ampicillin stock (100 mg/ml): weigh out 100 mg of ampicillin powder (Sigma), and dissolve in 1 ml of water. Filter through a 0.22-μm filter to sterilize and use at 100 μg/ml.

Antifoam: PPG-antifoam (Aldrich) 2 μl is added to 300 ml of culture media, before the induction of protein synthesis to prevent foaming and denaturation of proteins.

Competent (host) cells: many competent cell strains such as TOP™10 (Invitrogen), TOPP™ 6 (Stratagene) may be purchased from scientific suppliers or prepared as electrocompetent cells by the

*Xho*I

ggggtgtgtgatacgaaacgaagcattggcg*cctcgag*taatttaccaacactactacgttttaactgaaacaa

RBS *Nde*I

actggagact*cat* **ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT**

M K Y L L P T A A A

*Sfi*I *Nco*I *Msc*I

GGA TTG TTA TTA CTC GCG GCC CAG CCG GCC ATG GCC

G L L L L A A Q P A M A

*Bam*HI *Eco*RI *Sal*I

aa*ggatcc*taagtaagta*gaattc*tgagtaggtaa*gtcgac*aatcgcgtctagagccccgacgcgctggg

* * * * * *

Fig. 2. Nucleotide sequence over the promoter/RBS region and multiple cloning sites in the pPOW3 vector. Restriction sites for cloning are 5′; *Sfi*I (GGCCCAGC/CGGCC), *Nco*I (C/CATGG) or *Msc*I (TGG/CCA) and 3′ *Bam*HI (G/GATCC), *Eco*RI (G/AATTC) and *Sal*I (G/TCGAC). The codons for the PelB secretion signal are in bold, and the corresponding amino acids underneath. The translational stop codons are indicated with an asterisk.

method of Dower, 1990. Store at −70°C. The competent cells are thawed on ice then transformed with plasmid DNA, using the Bio-Rad Gene Pulser apparatus following the instructions of the manufacturer (12.5 kV/cm, 25 μF, 200 Ω). Note: do not heat shock the cells at any point as the vector is heat-inducible and will start protein synthesis.

3.8. Protein extraction reagents

Lysozyme extraction buffer: 20 mM Tris–HCl, pH 8.0, 0.2 mg/ml lysozyme (Sigma), 0.1% Tween 20 (polyoxyethylene–sorbitan monolaurate P-20 Sigma). Store at 4°C. Prepare on the day of use. The pH of this buffer may need to be altered depending on the p*I* of the expressed protein.

10×TBS stock (Tris buffered saline): 80.0 g NaCl, 2.0 g KCl, 30.0 g Tris base in 800 ml water. Adjust the pH to 8.0 with 1 M HCl. Adjust the volume to 1 l and sterilize by autoclaving. Store at room temperature. Dilute 1:10 for 1×TBS.

4 M urea/1×TBS: dissolve 240 g urea in a small volume of 1×TBS, then adjust volume to 1 l using more 1×TBS. Store at room temperature.

GEB (gentle elution buffer): 3 M $MgCl_2$, 20 mM MES buffer, pH 7.0, 0.25% Ethylene glycol (ethanediol). Store at 4°C.

Affinity resin: decide on the method of protein purification based on the tag tail and couple the appropriate affinity reagent to the resin (Harlow and Lane, 1988). In the case of a FLAG tail, bind the anti-FLAG antibody M2 (IBI) to the resin (Mini leak Low resin, Kem-en-tec) according to manufacturer instructions.

4. Detailed procedure

In order to synthesise protein from the pPOW3 vector we assume the gene of interest is correctly cloned, in-frame, re-creating the PelB signal sequence, (see support protocols Section 6.1 cloning of an antibody scFv into pPOW3) and has been freshly transformed into a bacterial host cell (see support protocols Section 6.2 scFv gene insertion, transformation and colony selection). We describe the protein synthesis and recovery followed by a brief

description of product stability and analysis of binding affinity to the target antigen.

4.1. Protein expression

4.1.1. Small scale screen to predetermine optimum induction time

Initially, a small scale (1 ml) screen of expression levels should be done as described in support protocols Section 6.4 to determine the optimum induction time for bacterial cell cultures.

4.1.2. Shake flask expression (1 litre) and cell fractionation

4.1.2.1. Preparation of starter culture. Use 2×YT +Ampicillin media. (see Section 3).

Day 1: (i) pick a colony from a YT+A plate; make a cell suspension using 1 ml of culture medium then use this to inoculate 100 ml of 2×YT+Amp media in a 1-litre flask. Incubate for a maximum of 16 h at 30°C.

4.1.2.2. Induction of protein synthesis. Day 2: (ii) autoclave and cool, four 1-litre flasks per sample, each containing 225 ml of 2×YT+Amp media. Use 25 ml of the overnight starter culture to inoculate each 225 ml of fresh 2×YT+Amp media in each 1-litre flask (A_{600} should be 0.5–0.8). Incubate with orbital shaking at 150–200 rpm and 30°C.

(iii) Monitor A_{600} at hourly intervals until it reaches mid to late exponential phase before induction. It usually takes about 4 h to reach an A_{600} of 4.0 (This is assuming a maximum OD of 7.0). Take a 1-ml 0-time sample as a preinduction control. Before inducing the sample add 2 μl of anti-foam (see Materials) to each flask.

(iv) Induce protein synthesis by increasing the temperature to 42°C to inactivate the temperature-sensitive repressor. Continue incubation at 42°C (with shaking as in (ii) above) for the optimal time determined from the small-scale experiment (see support protocols Section 6.4; usually 1 h for scFv dimers and 2 h for trimers), then reduce temperature to 20°C and continue incubation for one more hour.

(v) Spin down the samples in a refrigerated centrifuge at 4°C for 5 min at 6000 *g*. Freeze the cell pellet overnight. Discard the supernatant since this fraction is unlikely to have any of the recombinant protein product.

4.2. Protein fractionation (for 1 litre of original induced culture)

Days 3 and 4: (vi) thaw pellet and resuspend in 100 ml of lysozyme extraction buffer (see Materials). Leave on ice for 1 h. Cells will become thick and viscous.

(vii) Homogenise with an ultra turrex for 30 s to break up the DNA.

(viii) Spin for 10 min at 22 000 *g* in a refrigerated centrifuge at 4°C. Retain the aqueous phase (lysozyme wash fraction A). Freeze if affinity chromatography on this fraction is not done on the same day.

(ix) Resuspend the cell pellet in 100 ml of ice-cold sterile water. Leave on ice for 30 min. This step is the osmotic shock.

(x) Spin for 5 min at 22 000 *g*. Collect the aqueous phase, which contains the osmotic shock (fraction B), add 1/10th vol. of 10×TBS. Freeze if affinity chromatography on this fraction is not done on the same day.

(xi) Freeze the cell pellet. Slowly thaw the cell pellet, and then add 100 ml of ice-cold sterile water. Leave on ice for 30 min.

(xii) Spin for 5 min at 22 000 *g* in a refrigerated centrifuge at 4°C. Collect the aqueous phase (fraction C), add 1/10th vol. 10×TBS. Freeze if affinity chromatography on this fraction is not done on the same day.

(xiii) Fractions A, B and C should be analysed by SDS–PAGE to determine which fraction contains the greatest amount of protein (Sonication may be used to release more soluble proteins from the cells). If there is synthesised protein in all three fractions, A, B and C, can be combined as the total soluble fraction and affinity purified in one batch.

(xiv) The bacterial cell pellet contains the insoluble periplasmic proteins. Any proteins, which are to be recovered from the pellet, require solubilisation with denaturants such as urea or guanidine.

(xv) Resuspend the cell pellet in 50 ml of 4 M urea in TBS, pH 8.0. Leave on ice for 1 h (4 M urea solubilisations give better quality product with little or no aggregate after affinity chromatography,

whereas the 8 M urea solubilisations often result in 50% aggregate formation).

(xvi) Spin for 5 min at 22 000 *g* in a refrigerated centrifuge at 4°C. Load the sample into dialysis tubing (6000–8000 MW cut-off) and dialyse against 5×1 litre changes of TBS buffer for 16 h.

(xvii) Recover the solution from the dialysis tubing including a white precipitate that may have formed during dialysis. Spin the dialysed sample 5 min at 22 000 *g*. The aqueous phase contains the solubilised refolded proteins. The precipitate contains the insoluble material including misfolded scFv aggregates.

(xviii) Load the samples on two mini 12% glycine PAGE gels (Gradipore). Run at 150 V for 0.6 h. Typical volumes are as follows; Culture supernatant 20 μl, periplasm (osmotic shock) 10 μl, cell pellet 2 μl. After the run has finished Coomassie blue stain one gel (later destain to visualise the bands) and Western transfer the other (then probe the membrane with anti-FLAG M2 antibody and colour detect following manufacturers instructions).

4.3. Protein purification by affinity chromatography

Day 5: (xix) the affinity chromatography is performed using a batch method. The reason for this is to speed up the whole procedure and reduce the time available for bacterial proteases to degrade the synthesised protein. Separate batch chromatography steps were performed for the proteins from the soluble fraction and those from the urea refolded fraction.

(xx) Place the fractions containing the expressed protein into a 2-litre flask. Add 2 ml of affinity resin (see Materials) and gently swirl on a bench top shaker at 4°C for 1 h. Purify the protein samples by preadsorbing to an affinity matrix in a batch method rather than by using columns. The batch method is a lot faster and results in better protein product of the correct size. Keep the samples cold (4°C) at all times. Gently swirl the affinity resin during the binding phase, do not use magnetic stirrers, as these tend to be too vigorous in their action causing protein denaturation.

(xxi) Filter the mixture through a sintered glass funnel under vacuum. Wash the resin with 10 ml of 1×TBS, while still under vacuum.

(xxii) Release the vacuum and wash the resin into the column. Allow the resin to settle and pack. Connect the column into an FPLC system (e.g. Bio-Rad Econo system) and wash the column with 1× TBS (see Materials) Turn on the UV lamp and allow a stable base line to establish. Elute the bound protein with GEB buffer. Monitor the A_{280} absorbance and collect the peak.

(xxiii) Run a sample of the eluted protein on FPLC size exclusion columns (e.g. Superose 12) to determine the size (Holliger et al., 1993; Kortt et al., 1997; Atwell et al., 1999). More precise measurement of molecular mass can be determined by analytical ultracentrifugation.

4.4. Protein product analysis — stability and binding affinity

ScFv with linkers of 12 or more residues preferentially form monomers (~30 kDa), but have a propensity to dimerise into diabodies (~60 kDa). ScFv with 3–12 residues preferentially form diabodies and with <3 residues form triabodies (~90 kDa) or higher aggregates (Hudson and Kortt, 1999). Expression yields can be enhanced by point mutations or by codon usage (Ernst, 1988). Some scFv antibodies can be synthesised at higher yields due to their intrinsic solubility. Several key residues have been identified as being important for increasing the levels of soluble expression, particularly V_H residue 6 should be Glu, V_H position 84 Asp (Nieba et al., 1997; Hudson, 1999). Gene expression can be affected by codon usage and replacement of codons favoring those of *E. coli* preferred should be considered (23) and by choice of fermenter conditions (Kortt et al., 1995). Protein samples should be checked for stability on concentration as greater than 1 mg/ml can cause aggregation (Kortt et al., 1997; Arndt et al., 1998). Preferred storage is frozen at −20 or −80°C, providing that freeze–thaw cycles do not cause aggregation or unfolding. Alternatively, store in 0.02% azide at 4°C. Check size by size-exclusion FPLC, and check binding affinity to (immobilised) target antigen by ELISA or with BIAcore/IAsys biosensors.

5. Results

This bacterial expression system is heat induced. At 42°C the temperature sensitive promoter repressors are inactivated allowing the synthesis of recombinant protein. In a shaking incubator, it may take 45 min or more for the culture temperature to increase to 42°C required for induction (measure fluid temperature with a thermometer). Start timing the induction once this temperature has been reached. If optimal induction is at 42°C for 1 h, reduce the temperature to 20°C for a further 1 h maximum. The reason for this is that the bacterial cells will still be in a rapidly dividing state during protein synthesis and we have found that a further 1 h incubation at 20°C allows sufficient time for the last generation of bacterial cells to produce protein which greatly increases the overall protein yield. This method is unlikely to allow secretion of the protein into the supernatant.

Cell division (OD levels) and product synthesis continues for about 4 h post induction. It is unlikely that more than 4-h cultures are needed post-induction since we have observed no additional accumulation of the synthesised product after an overnight culture. Pay close attention to the bacterial cell A_{600} OD units for induction since protein synthesis should be done in the late stage of exponential growth.

Small amounts of protein can be recovered by affinity chromatography directly from the culture–supernatant if long induction times are used. This is likely to be due to cell rupture or lysis and is not detected in the early time points of the rapidly growing bacterial cell population. Therefore, long induction times can lead to cell death as the culture will be in stationary phase which increases the amount of protein recoverable from the culture supernatant.

The secretion signal PelB is efficiently excised after export through the inner membrane into the periplasm. By SDS–PAGE, there should be a predominant product in total cell extracts. A minor product ~2 kDa larger can be due to unprocessed precursor containing the PelB leader, but this contaminant is usually lost during purification since the hydrophobic PelB leader causes aggregation.

Periplasmic product may be either retained in the periplasm or secreted through the outer membrane into the culture supernatant. Relative levels in each compartment can be assessed by SDS–PAGE after fractionation. The periplasmic aggregates can be further separated into soluble components that are released by osmotic shock, and insoluble aggregates that require denaturation, purification and refolding. The optimum conditions for protein synthesis into each of these fractions can be controlled by the culture medium, the age of the bacterial population at time of induction and length of induction. At the highest level of protein synthesised, (50% of the total cell protein) one antibody V_H domain was predominantly in insoluble aggregates and could be recovered by a simple denaturation and dialysis refolding process to 30 mg/l in shake flasks (Power et al., 1992; Kortt et al., 1995). We have successfully used this basic protocol to produce scFv fragments either as monomers (Malby et al., 1993, 1998) or as multimers (Kortt et al., 1994, 1997; Iliades et al., 1997; Lawrence et al., 1998; Atwell et al., 1996, 1999). The significant gain in avidity (functional affinity) through multivalent binding makes multimeric scFvs attractive for in vivo tumour imaging since they exhibit improved pharmacokinetics compared to monovalent scFv (Wu et al., 1996; Zhu et al., 1996; FitzGerald et al., 1997; Adams et al., 1998). The gain in functional affinity for scFv dia/triabodies compared to scFv monomers is significant and is seen primarily in reduced off-rates (Kortt et al., 1997; Plückthun and Pack, 1997). However, it is difficult to accurately quantitate the kinetic constants for multivalent binding interactions in vitro, particularly using surface plasmon biosensors (Muller et al., 1998).

6. Discussion

(1) Trouble-shooting: all liquid and plated cell cultures prior to protein synthesis must be kept below 33°C, preferably 30°C. pPOW3 is a heat-inducible vector and to ensure good cell growth rates the repressor must be active and functional.

We have found that for protein synthesis it is best to choose a bacterial host cell strain that does not produce an *E. coli* protein at the same size as the expected synthesised protein. Use the following strains as a guide

Protein size (kDa)	Use cell strain
14	LE392 or SURE or TGI
27	LE392 or SURE or NM514
32	LE392 or SURE or NM514

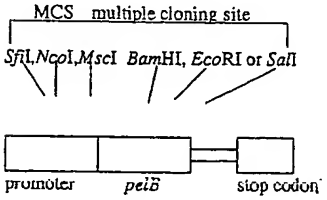
These cell strains produce slightly different *E. coli* banding patterns upon heat induction, when used for protein synthesis in conjunction with a cloned insert in the pPOW vector. Some cells are more susceptible to releasing the soluble proteins from the osmotic shock fraction. Try TOP 10 (Invitrogen), BL21, TOPP 6 (Stratagene) for greater cell permeability.

When you analyse the Coomassie Blue stained gel of the anti-FLAG affinity purified proteins and find additional proteins present, these can often be removed by modifying the method during the resin binding step, with the addition of 3 mM ATP to the TBS wash, before eluting the bound proteins. The ATP destabilises the non-specific binding to the resin.

(2) Alternative and support protocols

6.1. Cloning of an antibody scFv gene into pPOW3

The gene to be inserted into pPOW3 preferably encodes only the mature scFv protein. No leader or pre sequence is required as the pPOW3 vector provides the PelB secretion signal leader (Figs. 1 and 2). The 5′ end of the scFv gene should be designed for insertion into *Sfi*I or *Nco*I sites in the *pelB* gene to create the correct fusion to the encoded PelB leader sequence (Fig. 2). The 3′ end of the scFv gene should be designed for insertion into either *Bam*HI or *Eco*RI sites, which provide stop codons immediately downstream in all 3 reading frames. Only with insertion into the *Sal*I site is there a need to add an in-frame stop codon



6.1.1. Forward PCR primer

Cloning into the *Sfi*I or *Nco*I sites within the *pelB* gene: the forward PCR primer must create additional sequence 5′ to the scFv gene which encodes *pelB* so that insertion into *Sfi*I site (GGCCCAGC/CGGCC; 3-base 3′ overhang) or *Nco*I site (C/CATGG; 4 base 5′ overhang) within the *pelB* gene creates the exact PelB-scFv fusion protein. If using the *Nco*I site (shown in italics),

		*Sfi*I				*Nco*I	*Msc*I	
TTA	CTC	GCG	GCC	CAG	CCG	GCC	*ATG*	GCC
L	L	A	A	Q	P	A	M	A

The forward PCR primer must encode the *Nco*I site shown in italics and the additional nucleotides encoding (MA) added to the scFv, positioned immediately 5′ to the first codon of the scFv gene. *Nco*I cleaves C/CATGG, two amino acids away from the end of the *pelB* gene so to keep the translation in-frame for expression, these last amino acids (MA) will need to be reformed. These nucleotides can be easily incorporated in the PCR oligonucleotide primer used to assemble the gene fragment for subsequent cloning. For example, if the mature protein of the gene to be expressed begins with N-terminal Glutamine (CAG) and there are no *Nco*I sites in the gene, then the forward PCR primer would be designed as follows;

NcoI

5′ CAG CCG GCC *ATG* GCC CAG GTA CAG GTA CAG 3′ (30mer)

M A Gln

encoding CAG CCG GCC *ATG* GCC CAG GTA CAG GTA CAG

Q P A M A Gln Val Gln Leu Gln

pelB // foreign gene

When a PCR product made with this primer is cut with *Nco*I, the 5′ end of the gene will now be in-frame for expression. Extra nucleotides are added at the 5′ end of all of the PCR primers to help the restriction enzyme recognise its cleavage sequence. We prefer to have 50% of the primer binding to the template.

6.1.2. Reverse PCR primer

When the 3′ end of the foreign gene sequence encodes C-terminal arginine:

...CTG	GAA	ATC	AAA	CGT 3′
Leu	Glu	Ile	Lys	Arg

A restriction enzyme site (*Eco*RI) needs to be added and an optional tag tail. The reverse PCR primer would be designed as:

Tag tail

5′GTGC*GAATTC* XXX XXX XXX XXX ACG TTT GAT TTC CTG 3′

*Eco*RI Arg

A short polypeptide tail (encoding XXXX) can also be added to the C-terminus to help with the affinity purification if so desired. We typically use the FLAG sequence DYKDDDDK, which would be encoded by additional nucleotide sequence between the C-terminal (Arg) codon and the *Eco*R1 site in the example above. Other frequently used tag-tails for purification are hexa-his (Kipriyanov et al., 1997) and strep-tag (Schmidt and Skerra, 1994).

6.1.3. PCR amplification

Typical PCR cycle times for *pfu* polymerase are: (94°C for 2 min) 1 cycle, (94°C for 30 s, 55°C for 30 s, 72°C for 30 s) 35 cycles, (72°C for 2 min) 1 cycle, then 4°C hold. The PCR product should then be phenol extracted (to inactivate the polymerase), ethanol precipitated and resuspended in water. The DNA can be digested with the appropriate restriction enzymes (*Nco*I+*Eco*RI), then isolated from a gel (either agarose or acrylamide depending on the fragment size).

6.2. ScFv gene insertion, transformation and colony selection

The gel-purified PCR fragment can be ligated into *Nco*I+*Eco*RI digested pPOW3 vector and transformed into competent *E. coli* host cells using standard methods (Power et al., 1992; Malby et al., 1993). Use electrocompetent cells for transformations, but do not heat shock if using calcium competent cells as this will induce protein expression. The vector contains the Ampicillin resistance gene so YT+A plates should be used to select after transformation using suitable competent host cells. Positive transformants can be identified by colony screens using PCR with N2175 and N2357 primers, checking for a correct-size band by either acrylamide or agarose gel chromatography. If the vector contains no insert a PCR product of 215 bp will be amplified. This size represents the distance between the *Xho*I site, the *pelB* signal and the 3′ cloning site. If the transformant contains an insert, a PCR band of (the insert size) +215 bp will be amplified. With the selected clone, make a 100-ml cell culture in 1× YT+Amp media (30°C) and isolate plasmid DNA. The scFv gene insert can be sequenced using double-stranded DNA sequencing and the N2175 (forward) and N2357 (reverse) primers. The plasmid DNA stored frozen is the most stable stock of the expression vector. Use this stable plasmid DNA to transform competent host cells and select fresh colonies from YT+A plates for each protein synthesis experiment described below. Preferably use freshly transformed cells to ensure maximal plasmid copy number; and it is important to use cells or colonies that are only 2–3 days old at most.

6.3. SB (Superbroth) media, alternative bacterial cell growth media for antibody scFv protein synthesis

Take 20 g tryptone, 10 g yeast extract, 5 g NaCl, 2.5 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$ and make up to 1 l with water. Autoclave, cool, then add filtered stocks; 1 ml 0.1 mg/ml Biotin, 1 ml 1 mg/ml thiamine, 3 ml trace element solution, 1 ml 100 mg/ml Ampicillin. Trace element solution (100 ml) 1.6 g $FeCl_3$, 0.2 g $ZnCl_2 \cdot 4H_2O$, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g $CuCl_2$, 0.5 g H_3BO_3, 10 ml conc. HCl. Stir until dissolved. Adjust volume to 100 ml. Store frozen at −20°C. When this supplement is added to the medium, the medium will become slightly hazy. This does not affect the performance of the medium.

6.4. Small scale screen to predetermine the optimum induction time

In order to optimise synthesis of the desired proteins you should initially determine how long to induce the bacterial cultures. This small scale (1 ml)

screen will determine the induction time required. Once the temperature is raised to 42°C the temperature sensitive repressor is inactivated allowing the promoters to synthesise the foreign protein. For pPOW3, we find that optimal product synthesis occurs by continual repressor inactivation and concomitant protein synthesis using a constant temperature over 37°C (preferably 42°C). This experiment determines the optimal total induction time.

6.4.1. Preparation of starter culture

In the initial screening experiment where the product to be analysed is a total cell mixture including some culture supernatant, the samples should be prepared as follows; Use 2×YT +Ampicillin media (see Materials).

Day 1: (1) pick a colony from a YT+A plate (grown specifically for this experiment at 30°C). (2) Inoculate 1 ml of culture medium using a 10-ml tube. Incubate for a maximum of 16 h at 30°C.

6.4.2. Induction of protein synthesis (by heat shock)

Day 2: (3) use about 500 μl of the starter culture to inoculate 5 ml of 2×YT+Amp media in a 50-ml tube so that the A_{600} is between 0.5 and 0.8. The orbital shaker incubator speed should be set at 150–200 r.p.m.

(4) Incubate the cells for 4 h at 30°C in the orbital shaker (assume that it will take 4 h to reach an $A_{60}0$ of 4.0–5.0).

(5) Take a 0 time sample as a preinduction control (500 μl).

(6) To induce the culture increase the temperature to 42°C and hold at this temperature. Take 500 μl samples at the 1, 2, 3 and 4 h and overnight time points. Store samples on ice or at 4°C.

6.4.3. Determination of optimum time for induction

(7) Vortex time-point samples; take 30 μl and add 30 μl of SDS sample dye, boil and load 15 μl onto duplicate 12.5% SDS–PAGE gels, one gel for Coomassie Blue stain and the other for Western Blot followed by scFv-specific probing.

(8) After destaining, an induced Coomassie-stained band should be seen accumulating over time compared to the uninduced sample (0 time control), see Fig. 3.

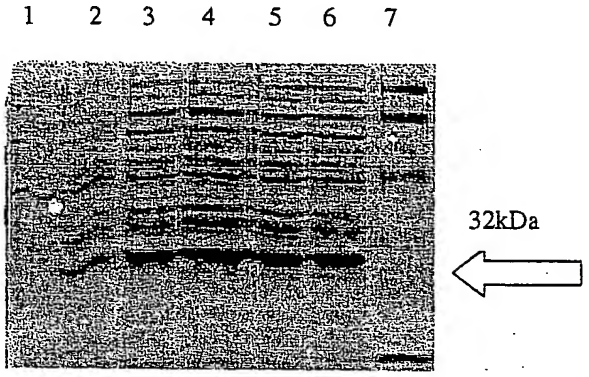


Fig. 3. A 12.5% SDS–PAGE gel of time course induction, stained with Coomassie Blue R-250. Each time point sample was thoroughly mixed to resuspend the cells in the culture medium. Then 15 μl of each time point sample was mixed with sample loading buffer, boiled, then loaded onto the gel. Arrow indicated protein band accumulating over time. Lanes: 1, preinduced; 2, 1 h postinduction; 3, 2 h postinduction; 4, 3 h postinduction; 5, 4 h postinduction; 6, 20 h postinduction; 7, low range molecular weight markers; 107, 74, 49, 36, 28, 21 kDa.

(9) Choose the earliest time point with high levels of expression.

7. Quick procedure for 1-l culture

7.1. Day 1 — starter culture

Start with an agar plate of freshly transformed cells. Pick a colony from a YT+A plate and inoculate 100 ml of 2×YT+Amp media in a 1-litre flask. Incubate for a maximum of 16 h at 30°C with shaking.

7.2. Day 2 — bacterial protein synthesis

Use the starter culture to inoculate 1 litre of fresh 2×YT+Amp media to A_{600} 0.5–0.8, divide into 4 1-litre flasks. Incubate at 30°C with shaking and monitor A_{600} at hourly intervals until mid to late exponential phase before induction (usually 4 h; ~A_{600} 4.0). Take a 1 ml zero-time sample as a

preinduction control. Add 2 μl of anti-foam to each flask.

Induce the culture by increasing the temperature to 42°C and incubate for 2 h with shaking, then reduce temperature to 20°C and incubate for one more hour. Spin down the samples in a refrigerated centrifuge at 4°C for 5 min at 6000 *g*. Discard the supernatant. Freeze the cell pellet overnight.

7.3. Day 3 — isolation of soluble proteins

Thaw the frozen cell pellet, resuspend in 100 ml of lysozyme extraction buffer and leave on ice for 1 h. Cells will become thick and viscous. Homogenise with an ultra-turrex at low speed for 1 min. Spin for 30 min at 22 000 *g* in the cold. Retain the supernatant (fraction A; lysozyme wash). Resuspend the cell pellet in 100 ml of ice-cold sterile water. Leave on ice for 30 min. Spin for 30 min at 22 000 *g*. Collect the supernatant (fraction B; osmotic shock). Freeze the cell pellet again and slowly thaw, then add 100 ml of ice- cold sterile water. Leave on ice for 30 min. Spin for 30 min at 22 000 *g*. Collect the supernatant (fraction C).

Combine fractions A, B and C and store at −20°C overnight. Retain and store the cell pellet at −20°C overnight since this contains the insoluble periplasmic proteins which are to be recovered by solubilisation with denaturants such as urea or guanidine.

7.4. Day 4 — affinity chromatography and refolding of denatured proteins

Resuspend the cell pellet in 50 ml of 4 M urea in TBS (pH 8.0). Leave on ice for 1 h. Spin for 30 min at 22 000 *g*. Load the supernatant into dialysis tubing (6000–8000 MW cut-off) and dialyse against 5×1-litre changes of TBS buffer for 16 h.

Recover the solution from the dialysis tubing and centrifuge for 5 min at 22 000 *g*. The aqueous phase (fraction D; urea refolded) contains the solubilised refolded proteins. Optionally analyse fractions by SDS–PAGE. Separate batch chromatography steps are performed for the proteins from the soluble fraction (A,B,C) and those from the refolded fraction D.

Place the fractions in a 2-litre flask, add 2 ml of affinity resin and gently swirl on a bench top shaker at 4°C for 1 h. Filter the mixture through a sintered glass funnel under vacuum. Wash the resin with 10 ml of 1×TBS, under vacuum.

Wash the resin into a column and connect into a FPLC system (e.g. Bio-Rad Econo system). Wash the column with 1×TBS and elute the bound protein with GEB buffer (see Section 3.4), monitoring the A_{280} absorbance and collect the major peak(s).

For molecular mass analysis, run a sample of the eluted protein on FPLC size exclusion columns (e.g. Superose 12) or, for more precise measurement, use analytical ultracentrifugation.

References

Adams, G.P., Schier, R., McCall, A.M., Crawford, R.S., Wolf, E.J., Weiner, L.M., Marks, J.D., 1998. Prolonged in vivo tumor retention of a human diabody targeting the extracellular domain of human HERk2/*neu*. Br. J. Cancer. 77, 1405.

Arndt, K.M., Muller, K.M., Plückthun, A., 1998. Factors influencing the dimer to monomer transition of an antibody single-chain Fv fragment. Biochemistry 37, 12918.

Atwell, J.L., Breheney, K.A., Lawrence, L.J., McCoy, A.J., Kortt, A.A., Hudson, P.J., 1999. scFv Multimers of the anti-neuraminidase antibody NC10: Length of the linker between V_H and V_L domains dictates precisely the transition between diabodies and triabodies. Protein Eng. 12, 101.

Atwell, J.L., Pearce, L.A., Lah, M., Gruen, L.C., Kortt, A.A., Hudson, P.J., 1996. Design and expression of a stable bispecific scFv dimer with affinity for both glycophorin and N9 neuraminidase. Molec. Immunol. 33, 1301.

Coia, G., Hudson, P.J., Lilley, G.G., 1996. Construction of recombinant extended single-chain antibody peptide conjugates for use in the diagnosis of HIV1 and HIV2. J. Immunol. Methods 192, 13.

Colcher, D., Pavlinkova, G., Beresford, G., Booth, B.J.M., Choudhury, A., Batra, S.K., 1998. Pharmacokinetics and biodistribution of genetically-engineered antibodies. Quart. J. Nucl. Med. 42, 225.

Dower, W.J., 1990. Electroporation of bacteria: a general approach to genetic transformation. Genet. Eng. NY 12, 275.

Harlow, E., Lane, D. (Eds.), 1988. Antibodies — A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Habor.

Ernst, J.F., 1988. Codon usage and gene expression. TibTech 6, 196.

FitzGerald, K., Holliger, P., Winter, G., 1997. Improved tumor targeting by disulphide stabilized diabodies expressed in *Pichia pastoris*. Protein. Eng. 10, 1221.

Holliger, P., Prospero, T., Winter, G., 1993. 'Diabodies': small bivalent and bispecific antibody fragments. Proc. Natl. Acad. Sci. USA 90, 6444.

Hudson, P.J., 1999. Recombinant antibody constructs in cancer therapy. Curr. Opin. Immunol. 11 (5), 548.

Hudson, P.J., Kortt, A.A., 1999. High avidity ScFv multimers; diabodies and triabodies. J. Immunol. Meth 231, 177.

Iliades, P., Kortt, A.A., Hudson, P.J., 1997. Triabodies: single chain Fv fragments without a linker form trivalent trimers. Febs. Lett. 409, 437.

Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S., Foeller, C., 1991. Sequences of Proteins of Immunological Interest. US Department of Health and Human Service, US Public Health service, NIH, Bethesda, MD.

Kipriyanov, S.M., Moldenhauer, G., Little, M., 1997. High level production of soluble single chain antibodies in small-scale *Escherichia coli* cultures. J. Immunol. Methods 200, 69.

Kortt, A., Malby, R., Caldwell, J., Gruen, L., Ivancic, N., Lawrence, M., Howlett, G., Webster, R., Hudson, P., Colman, P., 1994. Recombinant anti-neuraminidase single chain Fv antibody: characterization, formation of dimer and higher molecular mass multimers and the solution of the crystal structure of the scFv-neuraminidase complex. Eur. J. Biochem. 221, 151.

Kortt, A.A., Guthrie, R.E., Hinds, M.G., Power, B.E., Ivancic, N., Caldwell, J.B., Gruen, L.C., Norton, R.S., Hudson, P.J., 1995. Solution properties of *E. coli* expressed V_H domain of anti-neuraminidase antibody NC41. J. Protein Chem. 14, 167.

Kortt, A.A., Lah, M., Oddie, G.W., Gruen, L.C., Burns, J.E., Pearce, L.A., Atwell, J.A., McCoy, A.J., Howlett, G.J., Metzger, D.W., Webster, R.G., Hudson, P.J., 1997. Single chain Fv fragments of anti-neuraminidase antibody NC10 containing five and ten residue linkers form dimers and with zero residue linker a trimer. Protein Eng. 10, 423.

Lawrence, L.J., Kortt, A.A., Iliades, P., Tulloch, P.A., Hudson, P.J., 1998. Orientation of antigen binding sites in dimeric and trimeric single chain Fv antibody fragments. FEBS Lett. 425, 479.

Le Gall, F., Kipriyanov, S.M., Moldenhauer, G., Little, M., 1999. Di-, tri- and tetrameric single chain Fv antibody fragments against human CD19: effect of valency on cell binding. FEBS Lett. 453, 164.

Malby, R., Caldwell, J., Gruen, L., Harley, V., Ivancic, N., Kortt, A., Lilley, G., Power, B., Webster, R., Colman, P., Hudson, P., 1993. Recombinant anti-neuraminidase single chain antibody: expression, characterisation and crystallisation in complex with antigen. Proteins: Structure, Function Genet. 16, 57.

Malby, R.L., McCoy, A.J., Kortt, A.A., Hudson, P.J., Colman, P.M., 1998. Single-chain fv/neuraminidase complexes: Different stoichiometry in solution and crystals. J. Mol. Biol. 279, 901.

Muller, K.M., Arndt, K.M., Plückthun, A., 1998. Model and simulation of multivalent binding to fixed ligands. Anal. Biochem. 261, 149.

Nieba, L., Honegger, A., Krebber, C., Plückthun, A., 1997. Disrupting the hydrophobic patches at the antibody variable/constant domain interphase: improved in vivo folding and physical characterisation of an engineered scFv fragment. Protein Eng. 10 (4), 435.

Owens, R.J., Young, R.J., 1994. The genetic engineering of monoclonal antibodies. J. Immunol. Methods 168, 149.

Plückthun, A., Pack, P., 1997. New protein engineering approaches to multivalent and bispecific antibody fragments. Immunotechnology 3, 83.

Power, B.E., Ivancic, N., Harley, V.R., Webster, R.G., Kortt, A.A., Irving, R.A., Hudson, P.J., 1992. High-level temperature-induced synthesis of an antibody V_H -domain in *Escherichia coli* using the PelB secretion signal. Gene 113, 95.

Schmidt, T.G., Skerra, A., 1994. One-step affinity purification of bacterially produced proteins by means of the 'Strep tag' and immobilised recombinant core streptavidin. J. Chromatogr. A. 676 (2), 337.

Skerra, A., 1994. Use of the tetracycline promoter for the tightly regulated production of a murine antibody fragment in *Escherichia coli*. Gene 151 (1–2), 131.

Wu, A.M., Chen, W., Raubitschek, A., Williams, L.E., Neumaier, M., Fischer, R., Hu, S.-Z., Odom-Maryon, T., Wong, J.Y.C., Shively, J.E., 1996. Tumor localisation of anti-CEA single chain Fvs: improved targeting by non-covalent dimers. Immunotechnology 2, 21.

Wu, A.M., Williams, L.E., Zieran, L., Padma, A., Sherman, M., Bebb, G.G., Odom-Maryon, T., Wong, J.Y.C., Shively, J.E., Raubitschek, A.A., 1999. Anti-carcinoembryonic antigen (CEA) diabody for rapid tumor targeting and imaging. Tumor Targeting 4, 47.

Zhu, Z., Zapata, G., Shalaby, R., Snedecor, B., Chen, H., Carter, P., 1996. High level secretion of a humanised bispecific diabody from *E. coli*. Nature Biotechnol. 14, 192.